Tracking the dispersion of Scaphoideus titanus Ball (Hemiptera: Cicadellidae) from wild to cultivated grapevine: use of a novel mark–capture technique

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Abstract:	The dispersion of Scaphoideus titanus Ball adults was studied applying a water solution of cow milk (marker: casein) or chicken egg whites (marker: albumin) onto the canopy of wild grapevine at a distance from vineyards ranging from 5 to 330 m. Yellow sticky traps were placed on the canopy of grapes, and captured insects were analyzed via an indirect ELISA for markers' identification. Data were subject to exponential regression as a function of distance from wild grapevine, and to spatial interpolation (Inverse Distance Weighted and Kernel interpolation with barriers) using ArcGIS Desktop 10.1 software. The influence of rainfall and time elapsed after marking on markers' effectiveness, and the different dispersion of males and females were studied with regression analyses. Of a total of 5417 insects analyzed, 43% were positive to egg; whereas 18% of 536 tested resulted marked with milk. No influence of rainfall or time elapsed was observed for egg, whereas milk was affected by the time elapsed. Males and females showed no difference in dispersal. Marked adults decreased exponentially along with distance from wild grapevine and up to 80% of them were captured within 30 m. However, there was evidence of long-range dispersal up to 330 m. The interpolation maps showed a clear clustering of marked S. titanus close to the treated wild grapevine, and the pathways to the vineyards did not always seem to go along straight lines but mainly along ecological corridors. S. titanus adults are therefore capable of dispersing from wild to cultivated grapevine, and this may affect pest management strategies.

Tracking the ~~movement~~ dispersion of *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) from wild to cultivated grapevine: use of a novel mark-capture technique

Federico Lessio, Federica Tota, Alberto Alma

Abstract

The ~~movement~~ dispersion of *Scaphoideus titanus* Ball adults ~~from wild to cultivated grapevine~~ was studied ~~with a novel mark-capture technique~~applying~~.~~ a~~A~~ water solution of cow milk (marker: casein) or chicken egg whites (marker: albumin) ~~was applied directly~~ onto the canopy of wild grapevine ~~more or less in close proximity (5–350 m) to~~ at a distance from vineyards ranging from 5 to 330 m.~~;~~ Y~~y~~ellow sticky traps were placed on the canopy of grapes, and captured ~~S. titanus adults~~insects were analyzed via an indirect ELISA for markers' identification. Data were subject to exponential regression as a function of distance from wild grapevine, and to spatial interpolation ~~analyses~~ (Inverse Distance Weighted and Kernel interpolation with barriers) ~~were performed~~using ArcGIS Desktop 10.1 software.~~;~~ T~~t~~he influence of rainfall and time elapsed after marking on markers' effectiveness, and the different ~~dispersal patterns~~dispersion of males and females were ~~also~~ studied with regression analyses. Of a total of 5417 insects analyzed ~~for egg~~, 43% were positive to egg; whereas 18% of 536 tested ~~were milk~~ resulted marked with milk~~positive~~. No influence of rainfall or time ~~since the marker's application~~elapsed was observed for egg ~~marked specimens~~, whereas milk~~-marked were~~ was affected by the time elapsed. Males and females showed no difference in dispersal. Marked adults decreased exponentially along with distance from wild grapevine and up to 80% of them were captured within 30 m.~~;~~ H~~h~~owever, there was evidence of long-range dispersal up to ~~350~~ 330 m. The interpolation maps showed a clear clustering of marked

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S. titanus close to the treated wild grapevine, and the pathways to the vineyards did not always seem to go along straight lines but mainly along ecological corridors. *S. titanus* adults are therefore capable of ~~moving~~ dispersing from wild to cultivated grapevine, and ~~these new findings~~this ~~must be considered when deciding on~~may affect pest management strategies.

Key words: leafhopper vector, dispersal, immunomarking, ELISA, spatial interpolation

Introduction

The nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) was introduced into Europe in the late 1950s (Bonfils & Schvester, 1960) and is now widespread in many European countries from Portugal to Bulgaria (COST Action FA0807). This species is a grapevine specialist, and develops on both wild and cultivated grapevine (*Vitis* spp.). It is univoltine and overwinters in the egg stage, which is laid under the bark of wood 2-yrs of age or more (Vidano, 1964); eggs start to hatch in the middle of May and nymphs (which include five instars) are present until the end of July, whereas adults usually appear at the beginning of July and are observed up to the middle of October (Vidano, 1964). *S. titanus* is an important pest, as it is the main vector of grapevine's Flavescence dorée (FD), a disease caused by 16SrV phytoplasmas (subgroups C and D) (Malembic-Maher *et al.,* 2011). Nymphs from the 3rd instar on acquire phytoplasmas by feeding on infected plants (acquisition access period, AAP), and following a latency access period (LAP) of 4-5 weeks they become adults and able to transmit FD to healthy plants (IAP) (Bressan *et al.*, 2005). Since FD is a cause of great economic losses, insecticidal sprays against *S. titanus* are mandatory in Italy: active ingredients include neonichotinoids, organophosphates, etofenprox, and natural pyrethrum, the latter in organic farming (Lessio *et al.*, 2011a). However, there are still many ecosystems suitable to *S. titanus'* survival such as untreated vineyards, organic farming vineyards, cast-away vineyards, and woods or uncultivated areas colonized by wild grapevine (mainly from

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overgrown rootstocks: *Vitis rupestris, V. riparia* × *berlandieri*, etc.). Tthe easiest way to assess the threat of these areas to viticulture by serving as reservoirs for this leafhopper vector is to apply mark-release-recapture (MRR) or mark-capture (MC) techniques.

Marking methods used in entomology include fluorescent dusts (Garcia-Salazar & Landis, 1997; Takken *et al.,* 1998; Skovgard, 2002), radioisotopes (Hagler & Jackson, 2001), and immunomarking (Hagler & Jackson, 2001; Jones *et al.,* 2006; Hagler & Jones, 2010). In mark-release-recapture (MRR) experiments, insects (obtained under laboratory conditions or captured in the field) are marked, released at a certain point in the field, and then recaptured, usually by means of traps. However, there are many drawbacks in applying MRR methods, both generally and especially concerning *S. titanus*. First of all, it isn't possible to mark and release a quantity of insects as large as the effective population in the field. Mmoreover, the number of marked individuals recaptured is generally small, up to 8–10% (Zhou *et al.*, 2003; Lessio *et al.*, 2008). In addition, the marker may affect the insects' flight behaviour to some extent, and it is sometimes difficult to obtain a large quantity of insects, especially with species like *S. titanus* that have just one generation per year and an obligatory diapause and therefore cannot are difficult be to reared continuously under lab conditions. The possibility of applying a marker directly on the host plants overcomes these problems, and it is possible since the development of ELISA mark detection techniques. The first immunomarking method available was based on vertebrate proteins, such as chicken or rabbit immunoglobulin G (IgG) (Hagler, 1997; Blackmer *et al.,* 2004, 2006), but it hasn't been much used because it is too expensive. The development of low-cost markers, such as food proteins like cow milk, soy milk, or chicken egg whites, widened the possibility of using mark-capture techniques in entomology on large-scale experiments (Jones *et al.*, 2006). A recent study compared the performances of so-called first (IgGs) and second (food proteins) generation markers, and found that egg whites have a longer persistence than IgGs, whereas no difference was observed in the insects' mortality (Slosky *et al.*, 2012). For these reasons (the need to mark

78 field-born insect populations, low cost and high reliability of the markers), we decided to
79 apply this novel large-scale mark-capture technique to track the movements of *S. titanus*
80 adults from wild to cultivated grapevine in Northwestern Italy. As markers, we used cow milk
81 and chicken egg whites (see materials and methods for details).
82
83 ## Materials and methods
84 ### Large scale field marking and sampling of *S. titanus*
85 Field studies were conducted during 2010– and 2011 in the district of Portacomaro (AT),
86 Piedmont, Italy (44.97029-44.94596 °N, 8.24774-8.26120 °E). We set up four experimental
87 sites, called A, B, C and D; each site consisted of one or two vineyards (A-1 and A-2 for site
88 A, etc.) more or less in close proximity which disted from 5 to 330 m fromto woods colonized
89 by wild grapevine (WGV). All the vineyards were subject to insecticidal sprays: vineyard B
90 received two sprays with Etofenprox on the 26 June and 25 July, whereas all others were
91 sprayed with Thiamethoxam and Chlorpirifos-methyl on the first and second date,
92 respectively. Iin the middle of June, before the first spray, we assessed the presence of *S.*
93 *titanus* nymphs by visual inspection according to a sequential sampling plan with a fixed-
94 precision level of 75%, based on Green's equation (Lessio & Alma, 2006) (Table 1).
95 As markers we used albumin (pasteurized chicken egg whites: Eurovo SRL, S. Maria in
96 Fabiano Lugo, RA, Italy, approximate cost 5.00 €/lt.), and casein (sterilized Ultra High
97 Temperature, UHT cow whole fat milk: by Centrale del latte di Torino, Italy, approximate
98 cost 0.50 €/lt.), henceforth referred to as egg and milk, which have a greater reliability
99 compared to soy milk (Jones *et al.,* 2006). The markers were used as tap water solutions at a
100 ratio (volume/volume) of 10 and 20% for egg and milk, respectively; . Nowe didn't use any
101 water softener and/or wetting agent was used, as they don't significantly improve insect
102 marking in the field (Boina *et al.,* 2009). The markers were applied every 10–20 days from 8th
103 July to 10th September (Table 1) using a hand jet sprayer with a 15 l tank, at an approxa- rate

of 4000 l/~~100 m~~ha~~^{2}~~, directly onto WGV. When two separate WGV stands were present in the same site, we applied a different marker on each of them; otherwise, we applied only egg, which is more detectable than milk (Jones *et al.,* 2006). The daily amount of rainfall (mm) was recorded from a meteorological station ~~nearby~~set at the same distance (2 km) from each of the experimental sites.

Yellow sticky traps (cm 20 × 30) were placed in the vineyards at a distance of 15–20 ± 2 m from each other on the vine row, and 5-6 ± 0.5 m between rows, depending on plot size (for larger plots, we increased the distances in order to cover evenly the whole plot size), and directly on stands of WGV, at a distance of 15–20 ± 2 m from each other (Table 1; Figs. 3-6) to capture marked *S. titanus* adults; each trap was geo-referenced with a Garmin® GPS receiver and the distance between traps was confirmed by measuring with a graduated tape. Eight to 19 days after each marker's application, captured adults were carefully removed from the traps directly in the field using a wooden toothpick (using a new one every time to prevent cross-contamination), placed into sterilized 1.5 ml microcentrifuge tubes (one insect/tube), and stored at -20° C before analyses. The traps were placed at the beginning of July and replaced after each insect removal up to the middle of October, which represents the window of *S. titanus* adults' presence in North-western Italy (Lessio & Alma, 2004b).

Laboratory analyses

An indirect ELISA was performed to detect protein markers acquired by the leafhoppers; when egg and milk were used in the same sampling site, insects were analyzed so as to detect both markers at once. Commercially available antibodies for chicken egg albumin (RAE, (rabbit anti egg) (C6534, Sigma-Aldrich, St. Louis, MO, USA) and bovine casein (SAC, Sheep anti casein) (antibodies-online GmbH, Aachen, Germany) were used. The secondary antibodies used for the chicken egg albumin and bovine casein assays were peroxidase conjugated donkey anti-rabbit IgG (H + L) (DAR) (31458; Pierce Biotechnology, Rockford,

IL, USA) and peroxidase conjugated rabbit anti-sheep IgG (H + L) (RAS) (31480; Pierce Biotechnology, Rockford, IL, USA), respectively.

Reagents included: TBS-EDTA (Tris Buffered Saline, pH 8.0 plus 0.3 g/l sodium ethylenediamine tetra acetate) (Sigma-Aldrich, St. Louis, MO, USA); PBS-BS (Phosphate Buffered Saline + 20% Bovine Serum) (Sigma-Aldrich, St. Louis, MO, USA); PBSS-BS 20 (Phosphate Buffered Saline + 20% Bovine Serum + 1300 ppm Silweet L-77) (Silwet, Chemtura Manufacturing, Manchester, UK)); PBSS-BS 30 (Phosphate Buffered Saline + 30% Bovine Serum + 1300 ppm Silweet L-77); PBST (Phosphate Buffered Saline + 0.09% Triton X-100) (Triton-X-100; Sigma-Aldrich, St. Louis, MO, USA), PBS-SDS (Phosphate Buffered Saline + 2.3 g/l Sodium dodecyl sulfate), sulphuric acid (H_2SO_4) 2N; and immuno-pure ultra TMB substrate (Pierce Biotechnology, Rockford, IL, USA).

For the chicken egg assay, the primary antibody was diluted 1:4000 (2 µl in 8.0 ml) in PBSS-BS20, while the secondary antibody was diluted 1:6000 (1.4 µl in 8.4 ml) in PBSS-BS20.

For the casein assay, the primary antibody was diluted 1:500 (16 µl in 8.0 ml) in PBSS-BS30, while the secondary antibody was diluted 1:1500 (5.4 µl in 8.1 ml) in PBSS-BS20. The following protocol, slightly modified after Jones *et al.* (2006), was applied: 1 ml TBS-EDTA was added to the 1.5 ml microcentrifuge tube with the insect, vortexed for 2–4 seconds and left in stand-by mode for 3 minutes. From each tube, three 80 µl aliquots (replicates) were retrieved and placed in individual wells of a 96-well microplate (Nunc Polysorp, Nalge Nunc, Naperville, IL, USA) (to minimize contamination during washings, the 6 wells closest to the negative and blank controls were left empty); the micro-plate was then covered with aluminium foil and incubated at 37°C for 2 hrs. (at the end of this step, the leafhoppers were sexed by observing the external genitalia with a stereomicroscope and then discarded). The plate was then emptied and washed 5 times with 300 µl PBST using a LT-3000 micro-plate washer (Labtech International Ltd, Uckfield, UK);). Tthen 300 µl PBSS-BS (for egg) or 300 µl PBS-BS (for milk) were added, and the plate was incubated at 37°C for 1 hr. Afterwards, it

was washed 2 times with 300 µl PBST, and 80 µl of the first antibody (RAE for egg, SAC for milk) were added and the plate was incubated at 37°C for 30 min. The plate was then emptied, washed 5 times with 300 µl PBST, 80 µl of the second antibody (DAR for egg, RAS for milk) was added, and the plate was incubated at 37°C for 2 hrs. After incubation, the plate was washed 3 times with 300 µl PBS-SDS and 3 times with 300 µl PBST. Then 80 µl TMB were added and the plate was incubated at room temperature (25°C) in the dark on a shaker for 10 min. The reaction was then stopped by adding 80 µl of 2N H_2SO_4 and the plate was scanned with a LT-4000 micro-plate reader (Labtech International Ltd, Uckfield, UK) at wavelengths of λ=450 nm and 492 nm (reference standard).

As positive standards, we used adults of *Euscelidius variegatus* (Kirschbaum) (Hemiptera: Cicadellidae) reared on oat (*Avena sativa* L.) under laboratory conditions. Potted plants of either oat or broad bean (*Vicia faba* L.) were sprayed with the markers using a hand vaporizer, and then placed into insect-proof cages (cm 20 × 20 × 40) made of mesh and Plexiglas in a climatic chamber (T=23 ± 2 °C, RH=60%, L:D=16:8 h). In each cage (placed in the climatic chamber) we put ~~some~~ 90 *E. variegatus* adults; 7 days later, the leafhoppers were removed, killed by freezing, and preserved at -20° C before analyses; some untreated leafhoppers were used as negative controls, and extraction buffer alone was the blank control.

Each sample (=insect) was associated with 3 values of optical density (ODS) for each wavelength. The mean ODS at 450 was subtracted from the mean at 492: $ODS_{(450-492)}=ODS_{450}–ODS_{492}$; and the same equation was applied to the optical densities of the negative control: $ODN_{(450-492)}=ODN_{450}–ODN_{492}$; and blank: $ODB_{(450-492)}=ODB_{450}–ODB_{492}$. Finally, we obtained the corrected (blanked) optical density for each sample as: $ODCS=(ODS_{450-492})–(ODB_{450-492})$, and of the negative control as $ODCN=(ODN_{450-492})–(ODB_{450-492})$. A sample was considered marked when the ODCS was greater than the mean ODCN added plus 4 times its standard deviation (SD): ODCS>ODCN+4SD, providing additional protection against false positives (Jones *et al*., 2006).

182

183 **Data analyses**

184 The ~~movement~~ dispersion of *S. titanus* adults from WGV to the vineyards was studied by
185 fitting an exponential model: $N(r) = a \exp(-br)$, where N is the percentage of marked
186 individuals caught at the minimum distance r from the treated area (5 ± 1.5 m step), weighted
187 by the number of traps displayed at the same distance r (being P_i the number of positive
188 specimens captured on the total number of traps t_i placed at the i^{th} minimum distance r from
189 treated WGV, we have the grand total $T=\sum_i P_i/t_i$; and subsequently, we calculated $N=P_i/T$ as
190 the percentage of marked individuals per trap at the i^{th} distance r); a is a scaling parameter
191 that estimates the number of *S. titanus* collected at $r = 0$; and b is the spatial scale parameter
192 that models the rate of variation in insects captured. The choice of an exponential model was
193 made to verify if marked *S. titanus* would decrease at increasing distances from the source
194 (treated WGV) following an exponential decay pattern. For the same reason, for each
195 regression~~,~~ we calculated the median dispersal index $r_{0.5}$ (that is, the distance where 50% of
196 the marked individuals are found) using the negative half-life equation: $r_{0.5}=\ln(2)/b$
197 (Northfield *et al.*, 2009).
198 In order to assess differences in dispersal between genders, regression equations were
199 obtained separately for females and males and the homogeneity of the regression test was
200 evaluated (Sokal & Rohlf, 1995). The influence of rainfall occurred and time elapsed between
201 ~~since~~ the marker's application and insect sampling (independent variables) on the percentage
202 of positive individuals captured on traps placed within the treated points (dependent variable)
203 was studied by applying a weighted least square (WLS) linear regression, using the total
204 number of insects captured as the weight variable (Sokal & Rohlf, 1995). All regression
205 analyses were carried out with the SPSS 20.0® statistical package (http://www.spss.it).
206 ~~percentage~~ All percentage data were previously arcsin square root transformed.

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To individuate the pathways of *S. titanus* adults from WGV to vineyards, spatial interpolation of the marked insects captured was performed applying Inverse Distance Weighting (IDW) and Kernel interpolation with barrier (KB), both available in the ArcMap toolbox of ArcGIS Desktop 10.1 (http://esri.com). The choice of these two models rather than others was made in order to detect a movement pattern of *S. titanus* based solely on line of sight distances between sampling points (IDW), to another one that might be influenced by the presence of breaklines (KB). The IDW is a deterministic method, based on the Euclidean distance between sampling points (Bartier & Keller, 1996). It is easy and rapid to use, and is appropriate for aggregated data, as it highlights the hot spots (Tillman *et al.*, 2009). The generic IDW equation is: $z_{x,y} = \sum z_i w_i / \sum w_i$, where $z_{x,y}$ is the value to be estimated, z_i is the control value for the i^{th} sample point, and $w_i = (d_{x,y,i})^{-\beta}$ is the weight that states the contribution of each z_i in determining $z_{x,y}$, where d is the distance between sampling points $z_{x,y}$ and z_i, and β is defined by the user (the larger the value of β, the smaller the reciprocal influence of the sampling points; in this research we chose β=2, which is the most widely used). Kernel interpolation is used to determine the "utilization distribution" (UD) of a resource by an animal (Sheather & Jones, 1991; Benhamou & Cornélis, 2010). The ~~kernel~~ Kernel density estimate $\hat{f}_h$ of an univariate density f based on a random sample $X_1, \ldots, X_n$ of size n is: $\hat{f}_h(x) = n^{-1} \sum h^{-1} K[h^{-1}(x - X_i)]$, where K is the kernel function and h is the bandwidth, a smoothing parameter (Sheather & Jones, 1991). Kernel interpolation with barriers (KB) is a variant that uses a non Euclidean distance rather than a line of sight approach, so that the shortest distance between two points within the defined search neighbourhood is used to connect them; in this case, we used as Kernel function the exponential equation, which was used during the regression analysis (whereas no transfer function is needed to apply the IDW method)~~as kernel function~~, whereas the bandwidth was calculated as a default by ArcMap. Barriers were crops or natural vegetation stands between treated WGV and vineyards; however, they were considered partially open, as some

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movement within non-grapevine ecosystems may occasionally occur. The obtained interpolation maps were tested for accuracy via cross-validation: we calculated the mean prediction error: $ME=[\sum_{j=1,n} (\hat{x}_i - x_i)/n]$, and the root mean square error: $RMSE=\text{sqrt}[\sum_{j=1,n} (\hat{x}_i - x_i)^2/n]$, where $\hat{x}_i$ is the predicted value, x_i the observed value, and n the sample size. Both *ME* and *RMSE* are given in the same units of measure of the data: an ideal model should have a *ME* equal 0, and a *RSME* as small as possible. While *RMSE* gives an estimate of the error as a whole, *ME* mainly provides an estimate of the bias: that is, positive and negative *ME* values indicate that the model over or underestimates the data, respectively. (Rhodes *et al*., 2011).

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Results

In total, 1675 and 3901 *S. titanus* adults were captured in 2010 and 2011, respectively~~;~~. T~~t~~he flight peak occurred between the first ten days of August and the beginning of September. We analyzed 4881 insects by detecting egg alone (1664 in 2010 and 3217 in 2011), and screened 536 for both egg and milk (all in 2011). ~~The total net percentages~~ Without considering differences in sites and position of traps, ~~of~~ egg-positive individuals were 32 and 55% in 2010 and 2011, respectively (mean 43%). In 2010, the rate of egg-marked adults captured on WGV and in vineyards ranged from 36 to 44% and 9 to 68%, respectively (Fig. 1A~~);~~). H~~h~~owever, the minimum value of 9% refers to vineyard C-2, placed at a minimum distance of 220 m from the treated WGV, where few insects were captured. In vineyard B (minimum distance from WGV: Dmin.=6 m), although many insects were captured, there were few marked specimens (<~~40~~25%) ~~probably because of a high residential population of *S. titanus*; in fact, pest management in this site was different from (and probably less effective with respect to) the others~~ (Table 1). In 2011, we found 46–78% and 38–68% of egg-marked adults in WGV and vineyards, respectively (Fig. 1B). Milk was only used in site D in 2011 on one stand of WGV (Dmin.=110 m), whereas a second stand (Dmin.=120 m) was sprayed with egg: 97 (18%) of the 536 tested leafhoppers were milk-positive, and 82 of them were captured on

259 milk-sprayed WGV; 206 (38%) were egg-positive, and 131 were captured on egg-treated
260 WGV (Fig. 1B); finally, 58 (11%) of them were positive for both egg and milk at the same
261 time. The optical density values of positive specimens calculated on 5 plates chosen at
262 random (mean ± s.e.) were 0.67 ± 0.09 for egg, and 0.56 ± 0.19 for milk; positive reference
263 standards (*E. variegatus* maintained on treated broadbean or oat) scored 2.26 ± 0.03 for milk
264 and 2.28 ± 0.06 for egg, whereas negative controls (untreated *E. variegatus*) were 0.01 ± 0.00.
265 Rainfall occurred eight times both in 2010 (min. 1.4 mm, max. 35 mm, total amount 125
266 mm), and 2011 (min. 0.4 mm, max. 31 mm, total amount 67 mm). No influence of either
267 rainfall or time between applications was observed on the rate of egg-marked *S. titanus*; on
268 the other hand, milk-marked specimens were negatively related to time (Table 2).
269 The sex ratio (M/F) was generally female biased, both for total (0.39–0.55) and marked
270 (0.35–0.99) individuals; site C in 2010 represents an exception; it was investigated only from
271 the first week of August on, and the sex ratio was 0.08 for both total and marked insects. Egg-
272 marked specimens ranged from 33 to 66% for males, and 18–54% for females; whereas milk-
273 marked males and females were 17% and 19% of the total captured, respectively. The
274 homogeneity of regression test between the distribution of marked males and females as a
275 function of distance of capture from the treated point was never significant within different
276 experimental sites and years (Table 3). Therefore, the exponential models were fitted to the
277 experimental data (and the subsequent median dispersal indexes calculated) without taking
278 gender into account.
279 Exponential regression analyses provided a ~~good~~ significant fit of marked *S. titanus* adults as
280 a function of the minimum distance from the treated point, although in site D we obtained low
281 R^2 values; the subsequent median dispersal indexes ranged from 14 to 70 m within the
282 different experimental plots (Table 4). The cumulative distribution functions show how the
283 main captures (80%) occurred within 20–30 m from WGV (Fig. 2A, B:); however, there was
284 also evidence of long-range dispersal up to ~~350~~ 320 m (Fig. 2C, D). In site A, captures

decreased asymptotically after 25–30 m, although a slight increase was observed between 65 and 70 m (Fig. 2A), whereas in site B (investigated only during 2010) they were almost constant with increasing distance (Fig. 2B). In site C, in 2010 there was a clear point break (increase) at a distance of 30 m, and thereafter captures didn't increase anymore; but this site was only observed from the beginning of August in 2010. In the second vineyard (C-2), further from the treated zone, only a single marked specimen was captured. In 2011, the trend was smoother with a constant decrease in captures up to 60 m (maximum distance of the first vineyard, C-1, from WGV); up to 10% of the total marked insects were found in the second vineyard (C-2) (Fig. 2C). In site D, 70% of the egg-marked adults were captured on treated WGV and a cumulative 30% in the vineyard, at a 120–160 m distance, without any clear break point; on the other hand, only 60% of the milk-marked specimens were captured at the treated point, and 40% were found in the vineyard at a distance of 100–220 m (Fig. 2D).

On the whole, both IDW and KB interpolation methods showed a clear clustering of marked adults on the edges of the experimental vineyards. In many cases, when WGV was distributed along two edges, the clustering was much more evident if the European grapevine's rows were parallel rather than perpendicular to the edge, e.g. sites A (Fig. 3), and C, concerning the first vineyard (C-1) close to WGV (Fig. 5). Site B, only studied in 2010, shows almost the same pattern (Fig. 4); however, these results should be considered carefully because of the small size of the vineyard. In site D, egg and milk-marked individuals showed almost the same pattern independent of the interpolation method used (Fig. 6), ~~suggesting how an ecological corridor may exist between the two areas colonized by WGV~~. On the other hand, in site C long distance dispersal from the WGV to vineyard C-2 had a different pattern depending upon the interpolation method used: IDW produced a more uniform map, whereas KB showed how the possible ecological corridors are displaced along the rows (Fig. 5). On the whole, the cross-validation results showed lower ~~ME and~~ RMSE values for KB rather than for the IDW (with the exception of sites B and D, concerning egg-marked specimens),

indicating a better interpolation power of the first model compared to the second ~~interpolation method; the only exception was represented by egg-marked specimens in site D~~. The ME was generally positive for KB (overestimation) and negative (underestimation) for IDW, however KB always had a lower absolute value (the only exception was represented by egg-marked specimens in site D) (Table 5). Insects marked with both egg and milk were too few in number to perform cross-validation.

Discussion

The marking method proposed, used in large-scale application on *S. titanus*, was quite reliable with egg, as up to 78% of the insects captured on the traps placed into the treated wild grapevine (WGV) were marked; on the other hand, milk had a poorer performance (22%). These data are in accord with Jones *et al.* (2006), who obtained roughly 70% and 23% of marked *Cydia pomonella* L. in apple orchards treated with egg and milk, respectively; whereas Boina *et al.* (2009) obtained higher rates of *Diaphorina citri* Kuwayama marked with egg (88%) and milk (80%). In our research, one of the main problems was to properly treat the WGV canopy, as it develops up to 6 m above ground level in certain places and is sometimes very dense and difficult to reach. In order to study the movement of *S. titanus* during the entire period of the adults' presence in the field, we applied the markers constantly but sometimes with a longer window of time between application and the insects' removal from traps; otherwise, it would become too time-consuming. We found a higher rate of positive individuals in 2011, probably because of a smaller amount of rainfall~~;~~. H~~h~~owever, concerning egg, there was no influence of rainfall or time after the marker's application on the rates of positive individuals.; O~~o~~n the other hand, the time between application and removal did affect the rate of milk-marked *S. titanus*. In other researches, the rate of marked individuals decreased along with time after application and the amount of (simulated) rainfall (Jones *et al.,* 2006; Boina *et al.,* 2009). Under laboratory conditions, a residue egg-treatment

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337 on true bugs remained 68–100% positive up to 10 days after marking, and 27–88% positive
338 from 11 to 20 days after marking (Hagler & Jones, 2010). In addition, direct egg treatment of
339 *Hippodamia convergens* Guérin-Méleville allowed detection of egg proteins on 100% of the
340 individuals up to 26 days after marking (Sloski *et al.,* 2012). The problem with marking plants
341 is that insects must come into contact with the marker before it dries up or is washed off. In
342 addition, direct marking of *S. titanus* adults would not be reliable because of the difficulty in
343 obtaining a very large number of specimens, and we couldn't release this leafhopper in the
344 vineyards as it is subject to compulsory pest management. However, our data set (30–50% of
345 egg-marked specimens out of more than 5000 captured) seemed large enough to analyse and
346 interpret the movement patterns of this leafhopper vector.
347 *S. titanus* adults are therefore capable of both short and long range dispersal from wild
348 (WGV) to cultivated grapevine. This behaviour was previously theorized both in Italy (Pavan
349 *et al.,* 2012), and in the US (Beanland *et al.,* 2006) by comparing captures in traps placed at
350 different distances from potential *S. titanus* sources: the results of our mark-capture
351 experiments clearly demonstrate how these movements actually occur. The majority of
352 individuals seem to cover short distances: when WGV is close to the edge of the vineyards,
353 up to 80% of the marked individuals are captured within 30 m. However, long distance flight
354 is also possible: *S. titanus* captures on the local scale are spatially related up to 200 m,
355 whereas at greater distances they seem to depend on local factors, mainly pest management
356 strategies (Lessio *et al.,* 2011b). The results of this research confirm this aspect, as some
357 movement occurred up to more than 200 m. In vineyard B, although many insects were
358 captured, there were few marked specimens (<25%) probably because of a high residential
359 population of *S. titanus*; in fact, pest management in this site was different from (and probably
360 less effective with respect to) the others. Concerning site D, in the vineyard, the majority of
361 marked adults was captured in the North-West corner, suggesting how the infestation may
362 have mainly occurred from the second uncultivated area, treated with milk; however, this area

may also have recruited adults from other areas, as suggested by the double-marked individuals, and milk-marked adults being captured in the egg-treated zone and vice versa. On the whole, the Kernel with barriers (KB) interpolation method showed smaller errors (RMSE and absolute ME values) compared to inverse distance weighting (IDW): the first model, which derives partially from the exponential regression (used as a transfer function in the Kernel interpolation process) is therefore more accurate than the latter (due to lower RMSE values), and its overestimation of observed data (ME>0) has a lower absolute value than the underestimation given by IDW (ME<0).~~,~~ These differences suggest~~ing~~ how the movement patterns of *S. titanus* adults may not depend solely upon their distance from sources but also upon ecological corridors or natural barriers. It seems therefore that this leafhopper is less likely to perform direct long-distance flights, whereas it rather moves along more roundabout pathways. *S. titanus* adults have a crepuscular flight activity, which makes them not rely on the wind for dispersal (Lessio & Alma, 2004b), and this may be in accord with an active wandering movement rather than a passive wind-borne transport. Moreover, marked adults were generally clustered along the same row of cultivated grapevine rather than on different rows; this is in accord with the fact that they move mainly along the same row, and captures on the same row are more spatially related (Lessio *et al.,* 2009b). Males and females showed no differences in dispersal from wild to cultivated grapes. Usually, males of *S. titanus* start to fly earlier than females, however, in the late part of the season the presence and flight activity of females is increased, whereas males tend to decrease (Lessio *et al.*, 2009a). This long-range dispersion of females may have a consequence during the next year, resulting in a higher population of *S. titanus* in vineyards because of egg-laying.

Because WGV may also host 16SrV phytoplasmas (Lessio *et al*., 2007), incoming *S. titanus* adults may also be capable of transmitting FD to cultivated grapevine: in fact, symptomatic grapes are often clustered at the edges, consistent with *S. titanus* coming in from outside

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389 (Pavan *et al.,* 2012). Within this frame, pest management strategies against *S. titanus* in NW
390 Italy should be revisited, as the main problem seems to be represented by adults entering the
391 vineyards in the late part of the season; at present, PM focuses on a first spray against nymphs
392 at the end of June, a second one against adults at the middle-end of July, and a further one
393 sometime after harvest (Lessio *et al.*, 2011a). It is perhaps necessary to change this calendar,
394 using a more persistent active ingredient in the late part of the season to protect grapes from
395 inoculation; for instance, neonicotinoids are much more efficient than organophosphates in
396 preventing transmission (Saracco *et al.*, 2008).
397 Other strategies should be directed toward avoidance: the first action to be applied should be
398 to erase WGV as a source of *S. titanus*; however, such an action must not be done when adults
399 (both males and females) are present, as it may cause an increase of their movement onto
400 European grapevine. The same problem occurs when dealing with *Hyalesthes obsoletus*
401 Signoret, the vector of Stolbur phytoplasmas causing Bois Noir (Weber & Maixner, 1998),
402 which lives on weeds and only occasionally feeds on grapes as an adult (Alma *et al.,* 1987): if
403 weeds are erased, adults are compelled to move onto grapevine; for example, in Israel, where
404 *H. obsoletus* has two generations per year, the second generation is more likely to move to
405 grapes if its host plant is harvested or dries up because of summer heat (Orestein *et al*., 2003).
406 Another means of preventing leafhoppers from entering the vineyard may be the use of insect-
407 proof fences (nets). These devices were successfully used in Israel against some Diptera
408 (Vernon & MacKenzie, 1998; Päts & Vernon, 1999; Bomford *et al.,* 2000). A five metres
409 high screen barrier was successfully evaluated in Californian citrus orchards and nurseries
410 against *Homalodisca vitripennis* (=*coagulata*) (Say), a vector for *Xilella fastidiosa* causing
411 Pierce's disease (Blua *et al.,* 2005). Such a protective device against *S. titanus* should be at
412 least 2.5 m, as high as the flight boundary layer of this leafhopper (Lessio & Alma, 2004a).
413 Moreover, the screen should be provided with an overhang to avoid insects double crossing it
414 by walking on it (Bomford *et al.,* 2000). On the other hand, plantation of trees had

415 inconsistent effects in limiting invasion into vineyards by *Graphocephala atropunctata*
416 (Signoret), another vector for *X. fastidiosa* (Daugherty *et al.,* 2012).
417 In conclusion, the presence of wild grapevines in vine growing areas must be addressed with
418 an integrated pest management strategy that includes: area-wide sprays and use of suitable
419 active ingredients to prevent such transmission as much as possible; avoidance of new vine
420 plantations in regions with a high presence of WGV; destruction of WGV whenever possible,
421 which would decrease the pathways available to this leafhopper; and the development of new
422 tools such as physical barriers to avoid the entrance of *S. titanus* adults into vineyards from
423 outside.
424

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431

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Table 1. Main features of the experimental sites and marker -applications.

Site	Vin.	Coordinates (°N; E)	~~Cv~~Variety	S_V	Y_P	Y_S	STN	$D_{min.}$	N_V	N_{WGV}	N_m	AP
A	A-1	44.965299; 8.252597	Barbera	2780	2004	2010	0.05	6	29	6	5 *	Jul. - Sept.
						2011	0.14		29	4	8 *	Jul. - Oct.
	A-2	44.965215; 8.252018	Grignolino	1500	2008	2010	0.01	14	17	6	5 *	Jul. - Sept.
						2011	0.01		20		8 *	Jul. - Oct.
B	B	44.946083; 8.247651	Freisa	1800	1970	2010	0.31	6	19	4	5 *	Jul. - Sept.
C	C-1	44.970248; 8.252081	Barbera	2800	1981	2010	0.18	20	23	4	2 *	Aug. - Sept.
						2011	0.08		23	3	8 *	Jul. - Oct.
	C-2	44.968798; 8.249197	Barbera	2550	2004	2010	0.01	220	16	4	2 *	Aug. - Sept.
						2011	0.03		20	3	8 *	Jul. -Oct.
D	D	44.962938; 8.260826	Barbera, Grignolino, Ruché	8600	2008	2011		120	24	3	7 *	Jul. - Oct.
							0.05	110		2	7 **	Jul. - Oct.

Sites consisted of vineyards and stands of wild grapevine. All vineyards (Vin.) were treated with Thiametoxam (approx. 26 June) and Chlorpirifos-methyl (approx. 25 July), except vin. B that was treated twice with Etofenprox on the same dates; S_V: size of vineyards, in m^2; Y_P: year of planting; Y_S: year of study; STN: density of *S. titanus* nymphs /5 leaves per plant in the vineyard, calculated with a sequential sampling plan (Lessio & Alma, 2006). $D_{min.}$: minimum distance in metres from stands of wild grapevine (WGV); N_{WGV}: number of traps on stands of WGV (in site D there were 2 separate stands of WGV); N_V: number of traps in vineyards; N_m: number of markers' application during the season; *: egg; **: milk; AP: application period of markers during the season.

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Table 2. Results of weighted least square (WLS) regression of marked *S. titanus* as a function of rainfall and time.

Marker	Year	N	T	Independent variable	b	s.e	t	P
Egg	2010	5	24	Intercept	0.83	0.13	6.27	0.00
				Time	-0.01	0.01	-0.63	0.54
				Rainfall	-0.00	0.01	-0.91	0.38
	2011	8	17	Intercept	1.06	0.14	7.47	0.00
				Time	-0.01	0.01	-0.69	0.52
				Rainfall	-0.01	0.01	-0.70	0.51
Milk	2011	7	2	Intercept	-0.15	0.13	-1.21	0.29
				Time	0.04	0.01	2.99	0.04
				Rainfall	-0.01	0.01	-0.94	0.40

Dependent variable: rate of marked *S. titanus* (previously arcsin square root transformed) collected on traps placed on wild grapevine (WGV) at each observation, without considering differences between experimental sites; N: number of observations during the season; T: number of traps observed; independent variables: rainfall occurred (mm) and time elapsed (days) ~~from~~ between marker's application on WGV and insects' collection; weight variable: total insects captured (marked + unmarked) on traps placed on WGV at each observation.

Table 3. ~~H~~Sex ratios observed, and homogeneity ~~of regression~~ test for exponential regression of marked *S. titanus* males and females ~~*S. titanus*~~ captured at different distance from wild grapevine (WGV).

year	site	males		females		Sex ratio (m/f)		Homogeneity of regressions		
		total	marked	total	marked	total	marked	F	df	P
2010	A*	276	115	549	188	0.50	0.61	1.10	1, 21	0.31
	B*	255	85	4065	86	0.06	0.99	0.05	1, 7	0.83
	C*	12	4	151	51	0.08	0.08	0.81	1, 21	0.38
2011	A*	755	455	1377	739	0.55	0.62	0.17	1, 21	0.68
	C*	298	197	761	406	0.39	0.49	1.88	1, 23	0.18
	D*	150	92	386	171	0.39	0.54	0.18	1, 11	0.68
	D**	150	25	386	72	0.39	0.35	2.84	1, 11	0.12

Dependent variable: rate of marked *S. titanus* males and females (marked/total) previously arcsin square root transformed~~)~~; independent variable: distance from treated WGV. *: egg; **: milk; df: degrees of freedom.

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Table 4. Results of exponential regression of marked *S. titanus* adults as a function of minimum distance from wild grapevine (WGV).

year	site	intercept	slope	R^2	P	$r_{0.5}$
2010	A*	8.27	0.05	0.56	<0.05	13.86
	B*	9.51	0.03	0.48	<0.05	23.10
	C*	73.43	0.04	0.61	<0.05	17.33
2011	A*	55.69	0.05	0.80	<0.05	13.86
	C*	4.19	0.02	0.84	<0.05	34.66
	D*	29.13	0.01	0.34	<0.05	69.31
	D**	6.2	0.01	0.12	<0.05	69.31

Dependent variable: percentage of marked *S. titanus* captured during the whole season at the same minimum distance from treated wild grapevine (WGV), weighted by the number of traps placed at the same distance ~~per trap~~; independent variable: minimum distance from treated ~~wild grapevine (~~WGV~~)~~ (see text for details). *: egg; **: milk; $r_{0.5}$: mean dispersal index (in metres).

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Table 5. Results of cross-validation analysis on the interpolation maps of marked *S. titanus* adults.

year	site	interpolation method	ME	RMSE
2010	A*	IDW	-1.27	7.85
	A*	KB	0.70	6.51
	B*	IDW	-1.06	5.58
	B*	KB	0.70	5.73
	C*	IDW	-0.72	1.51
	C*	KB	0.22	1.20
2011	A*	IDW	-4.48	42.90
	A*	KB	-0.88	14.23
	C*	IDW	-2.38	14.12
	C*	KB	0.31	12.71
	D *	IDW	-1.54	15.26
	D *	KB	2.32	19.26
	D **	IDW	-0.39	6.18
	D **	KB	0.21	2.70

*: egg; **: milk; IDW: Inverse Distance Weighting; KB: Kernel interpolation with Barriers; ME: Mean Error; RMSE: Root Mean Square Error.

Figure captions

Fig. 1. Captures of *Scaphoideus titanus* adults on stands of wild grapevine (WGV) and in vineyards within the different experimental sites, and rate of marked specimens (*: egg; **: milk). A: 2010; B: 2011.

Fig. 2. ~~Cumulative distribution frequencies~~Frequencies (F) and cumulative frequencies (CF) of marked *Scaphoideus titanus* adults ~~(CF marked)~~ as a function of minimum distance (Dmin) from treated stands of wild grapevine (WGV) in the different experimental sites: A: site A (vineyards A-1 and A-2 + 1 WGV); B: site B (vineyard B + 1 WGV); C: site C (vineyards C-1 and C-2 + 1 WGV close to C-1); D: site D (vineyard D + 2 WGV); *: egg; **: milk.

Fig. 3. Interpolation maps of marked *Scaphoideus titanus* captures in site A. IDW: inverse distance weighting; KB: kernel interpolation with barriers. A: IDW, 2010; B: IDW, 2011; C: KB, 2010; D: KB, 2011. Dots represent the position of yellow sticky traps (sampling points).

Fig. 4. Interpolation maps of marked *Scaphoideus titanus* captures in site B. IDW: inverse distance weighting; KB: kernel interpolation with barriers. A: IDW, 2010; B: KB, 2010. Dots represent the position of yellow sticky traps (sampling points).

Fig. 5. Interpolation maps of marked *Scaphoideus titanus* captures in site C, IDW: inverse distance weighting; KB: kernel interpolation with barriers. A: IDW, 2010; B: IDW, 2011; C: KB, 2010; D: KB, 2011. Dots represent the position of yellow sticky traps (sampling points).

Fig. 6. Interpolation maps of marked *Scaphoideus titanus* captures in site D, obtained with Inverse distance weighting (IDW) or kernel interpolation with barriers (KB). A: IDW, egg,

2011; B: IDW, milk, 2011; C: IDW, egg + milk, 2011; D: KB, egg, 2011; E: KB, milk, 2011; F: KB, egg + milk, 2011. Dots represent the position of yellow sticky traps (sampling points).

Figure 1 A

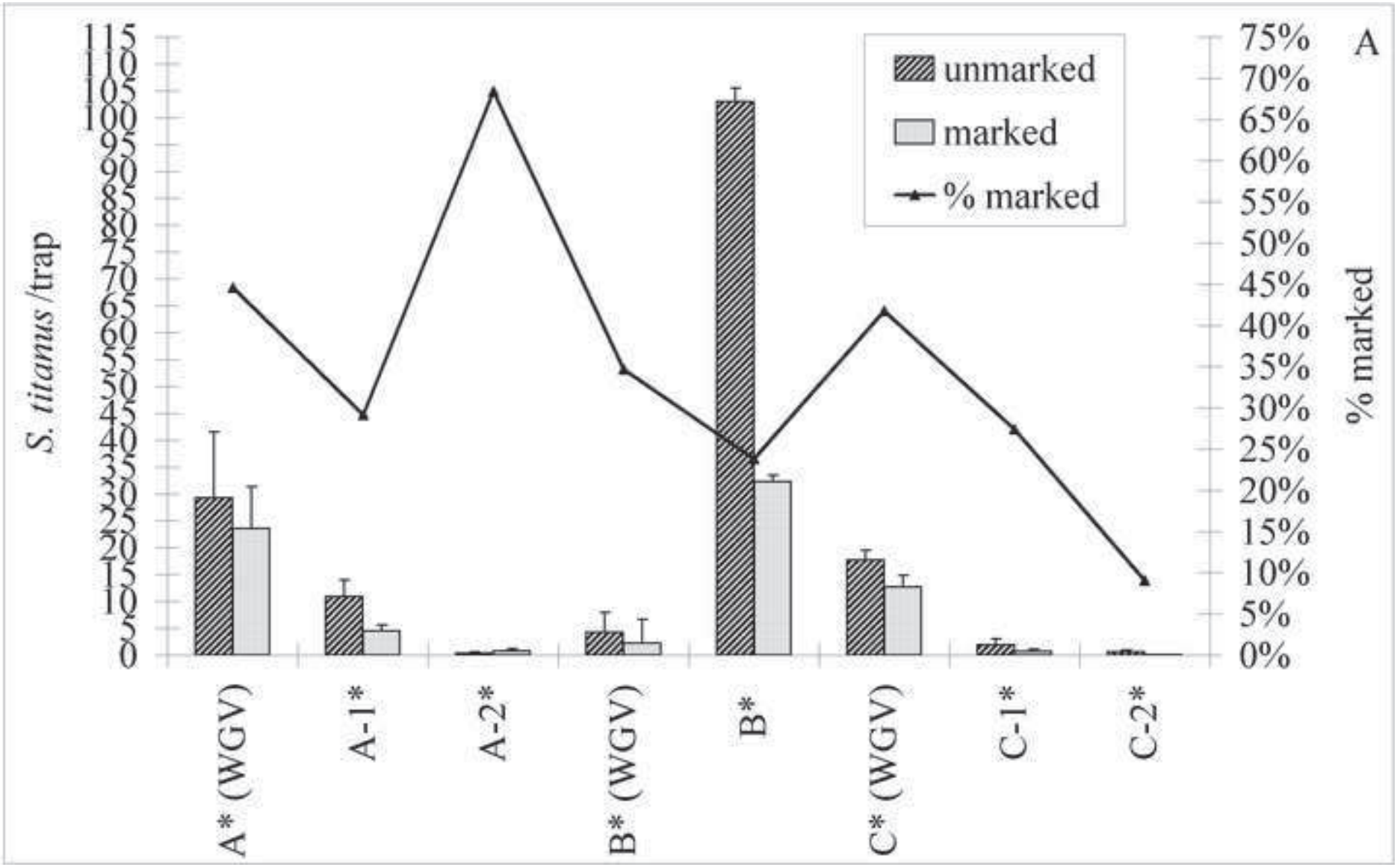

A
S. titanus /trap
% marked
unmarked
marked
% marked
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75%
A* (WGV)
A-1*
A-2*
B* (WGV)
B*
C* (WGV)
C-1*
C-2*

Figure 1 B

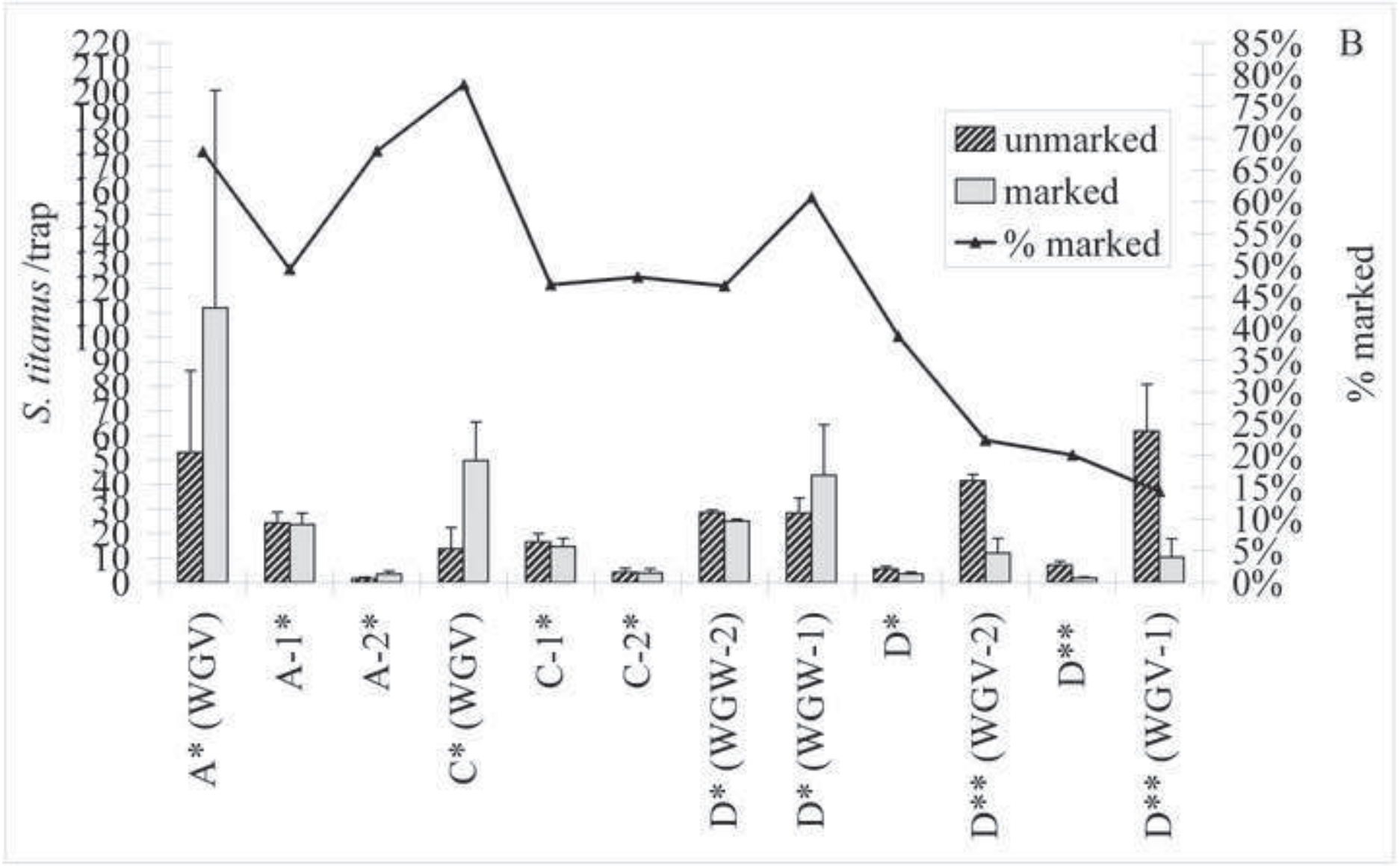

B
S. titanus /trap
% marked
unmarked
marked
% marked
A* (WGV)
A-1*
A-2*
C* (WGV)
C-1*
C-2*
D* (WGW-2)
D* (WGW-1)
D*
D** (WGV-2)
D**
D** (WGV-1)

Figure 2 A

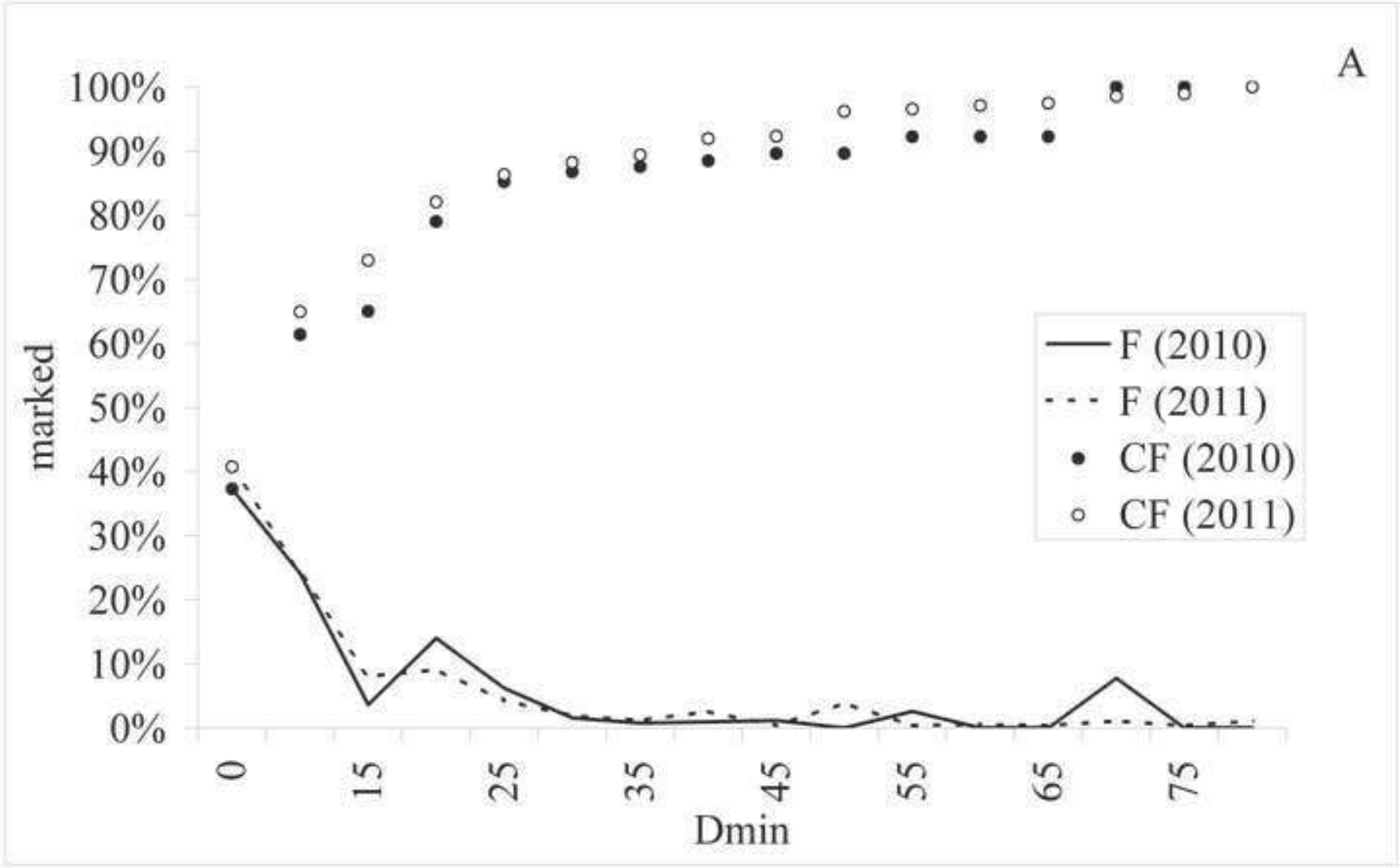

A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
marked
0
15
25
35
45
55
65
75
Dmin
F (2010)
F (2011)
CF (2010)
CF (2011)

Figure 2 B

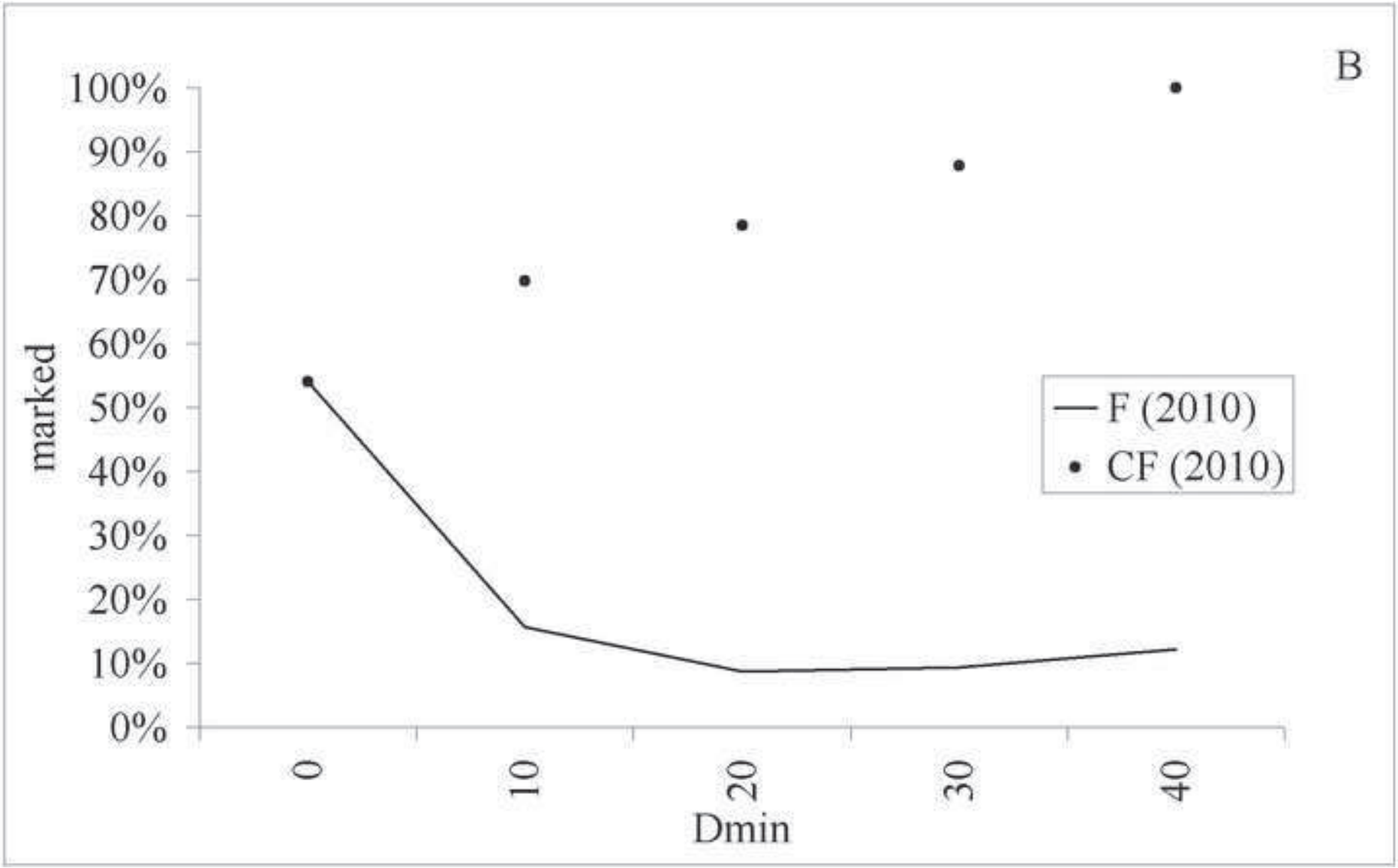

B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
marked
0
10
20
30
40
Dmin
F (2010)
CF (2010)

Figure 2 C

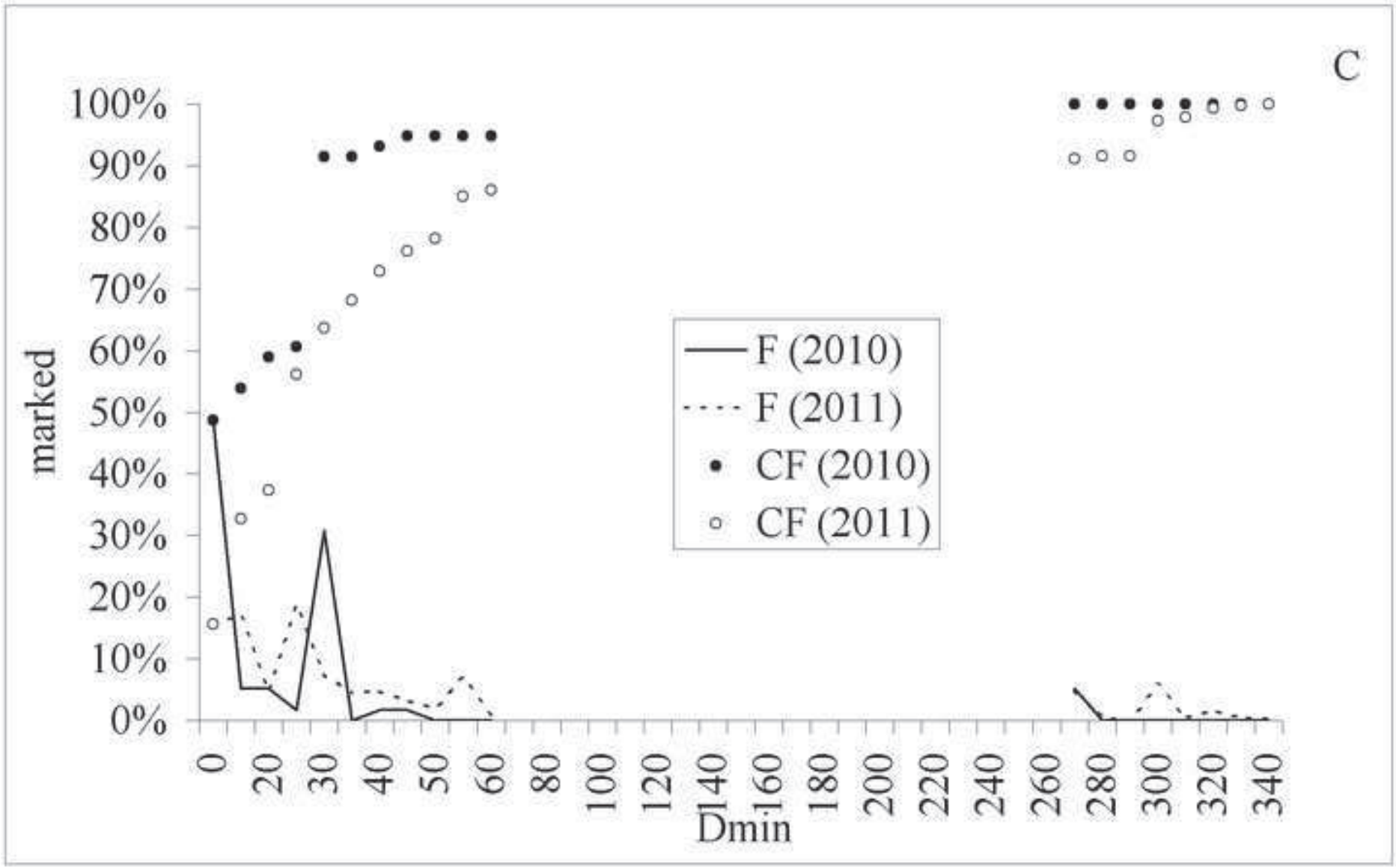

C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
marked
F (2010)
F (2011)
CF (2010)
CF (2011)
0
20
30
40
50
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
Dmin

Figure 2 D

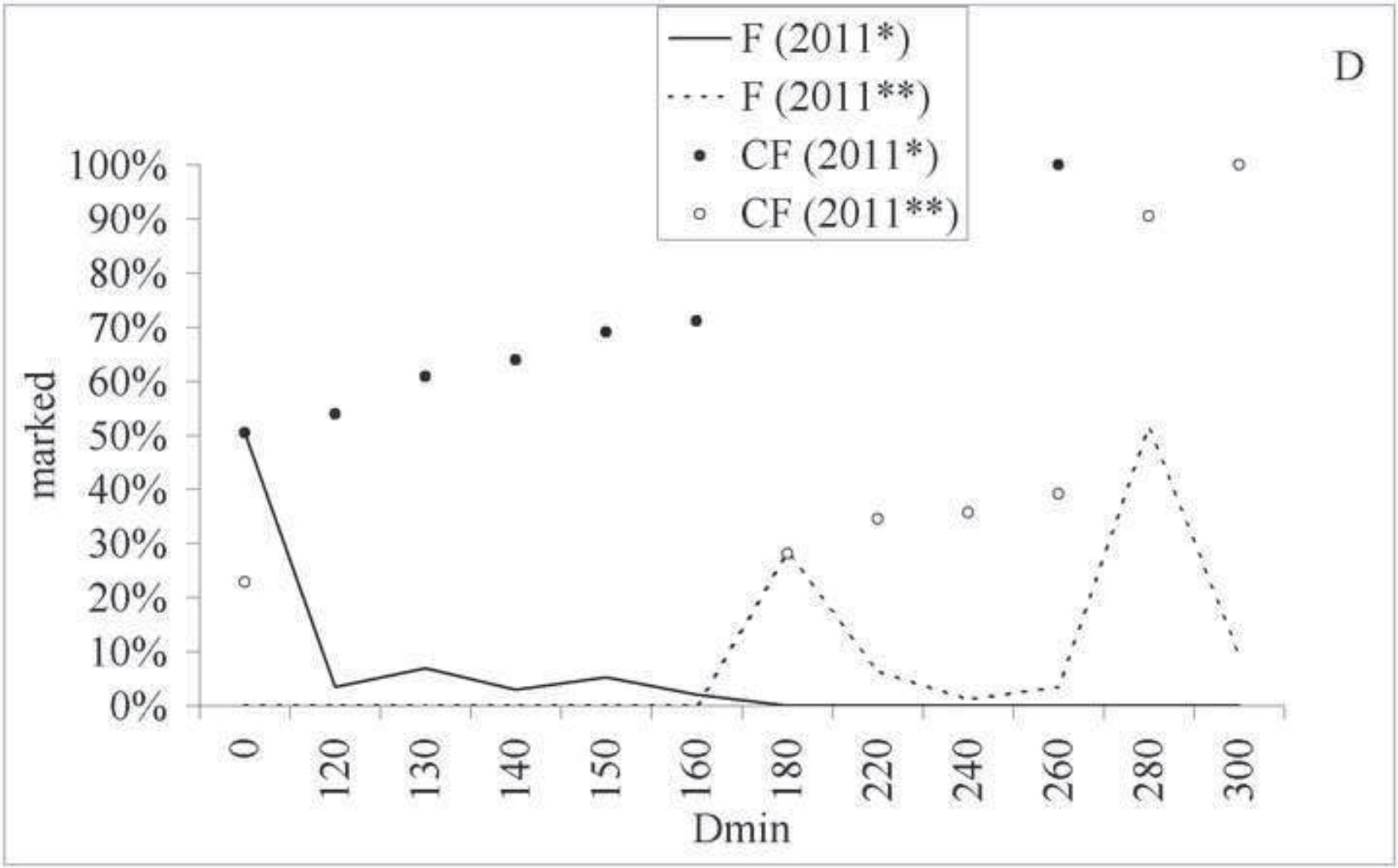

F (2011*)
F (2011**)
CF (2011*)
CF (2011**)
D
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
marked
0
120
130
140
150
160
180
220
240
260
280
300
Dmin

Figure 3

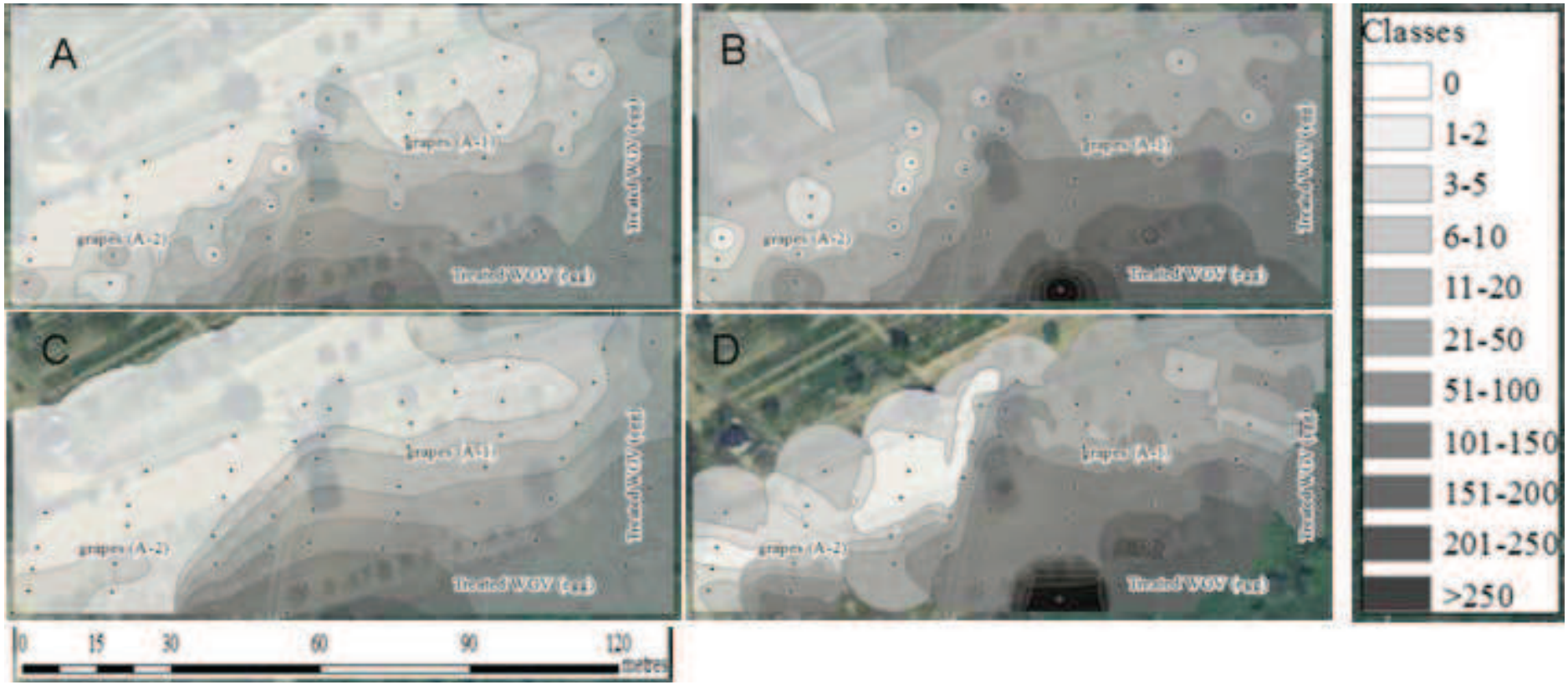

A
B
C
D
Classes
0
1-2
3-5
6-10
11-20
21-50
51-100
101-150
151-200
201-250
>250
0
15
30
60
90
120
metres

Figure 4

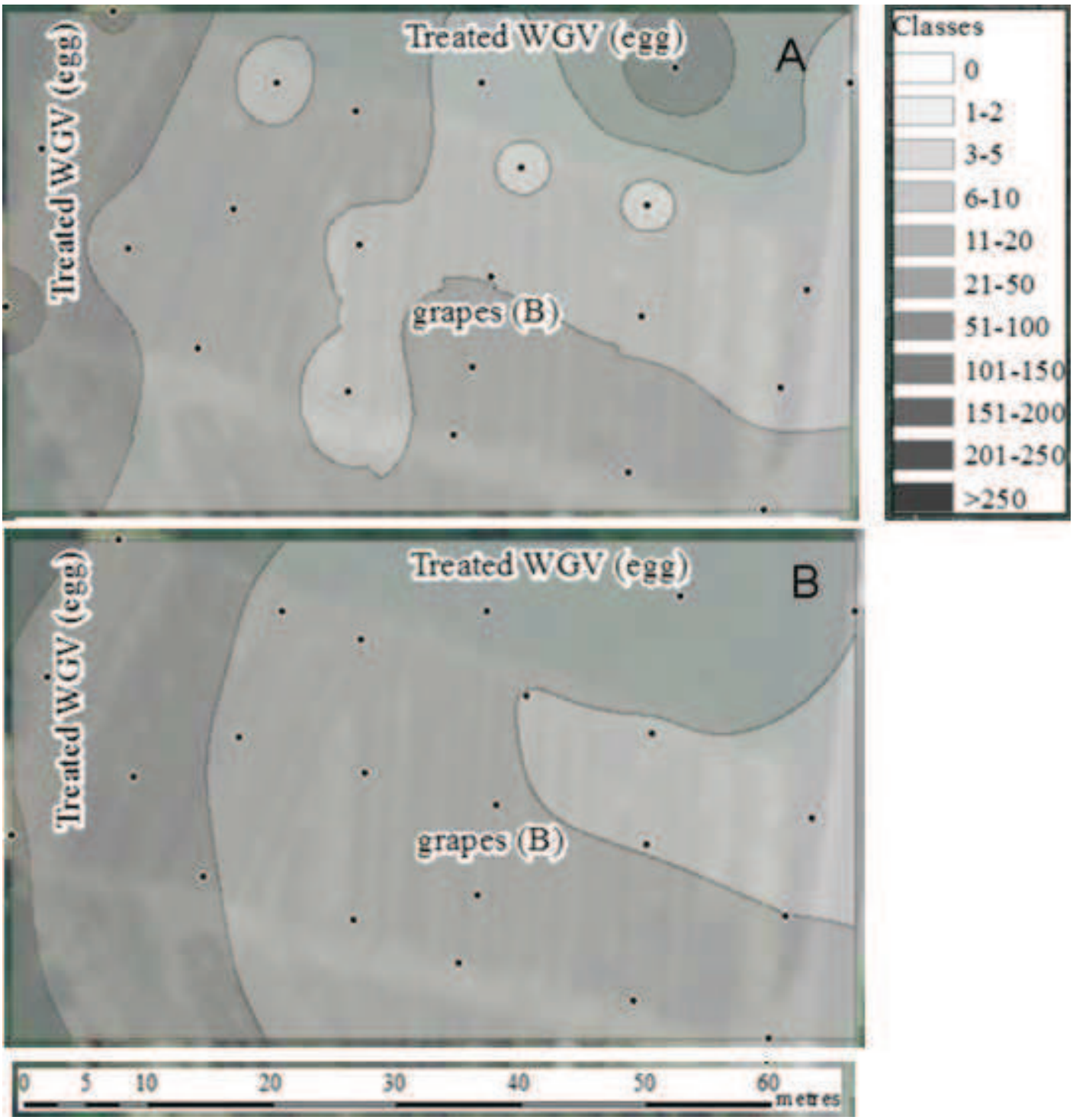

Treated WGV (egg)
A
Treated WGV (egg)
grapes (B)
Classes
0
1-2
3-5
6-10
11-20
21-50
51-100
101-150
151-200
201-250
>250
Treated WGV (egg)
B
Treated WGV (egg)
grapes (B)
0 5 10 20 30 40 50 60
metres

Figure 5

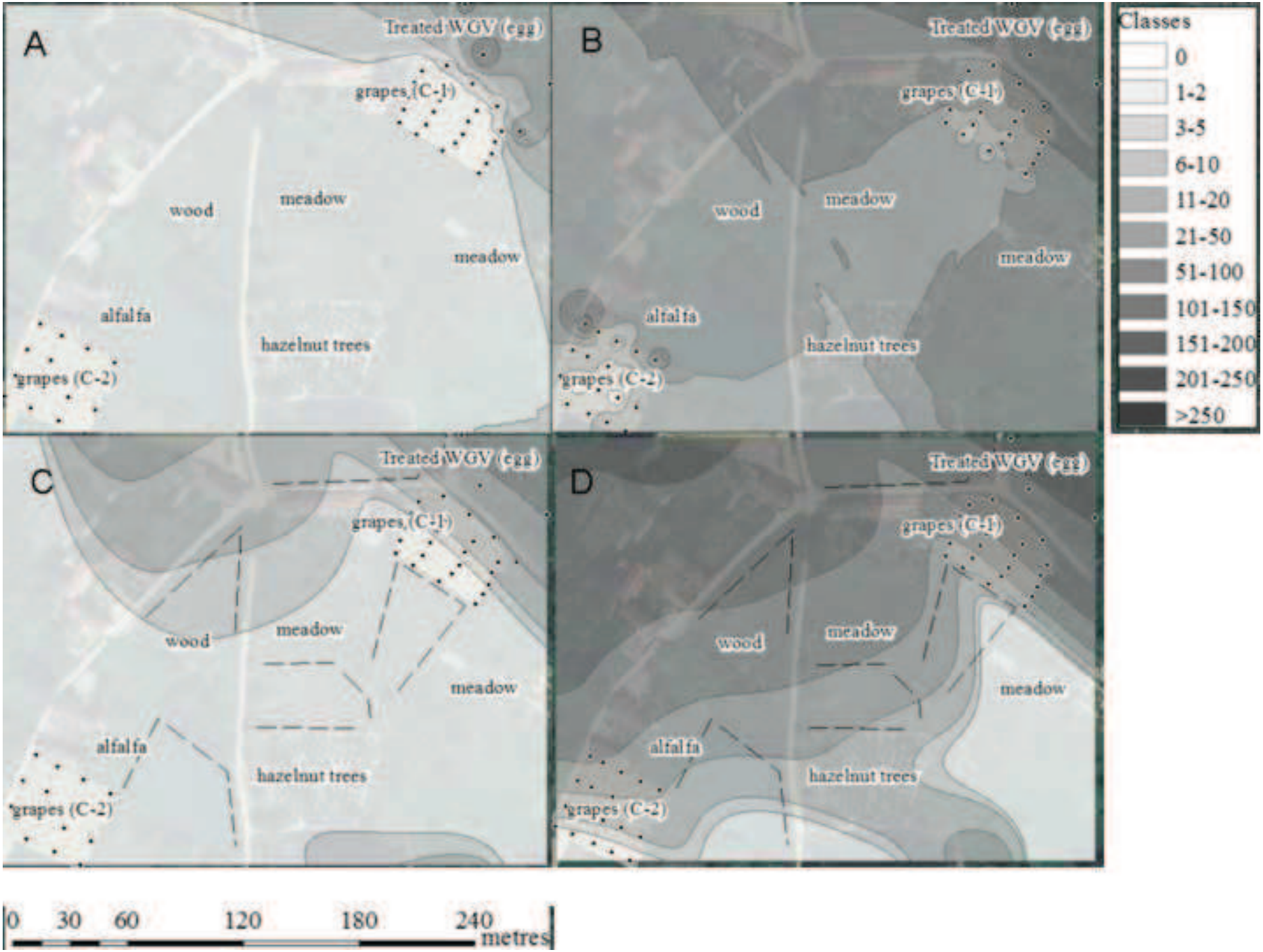
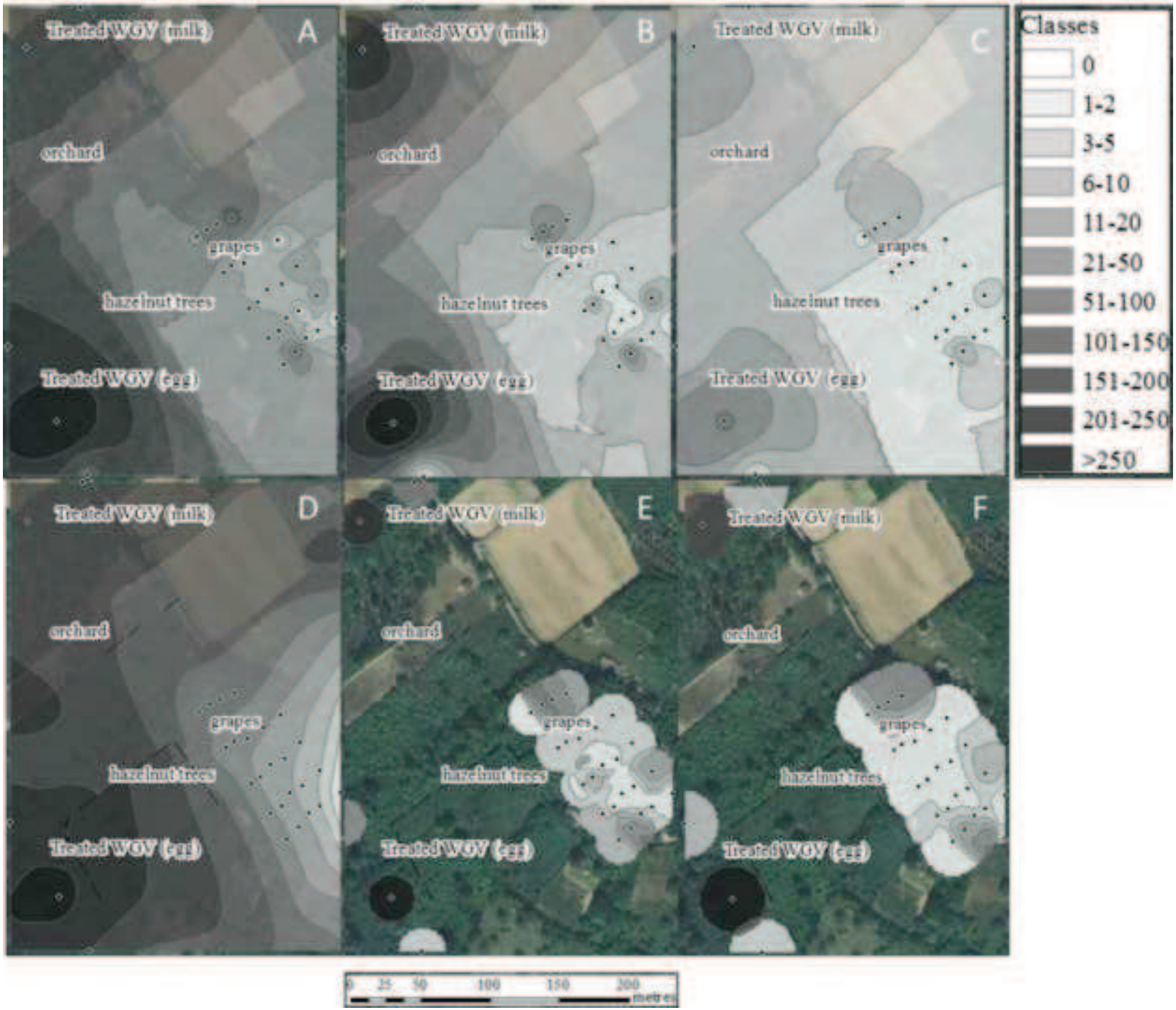

A
Treated WGV (egg)
grapes (C-1)
wood
meadow
meadow
alfalfa
hazelnut trees
grapes (C-2)
B
Treated WGV (egg)
grapes (C-1)
wood
meadow
meadow
alfalfa
hazelnut trees
grapes (C-2)
Classes
0
1-2
3-5
6-10
11-20
21-50
51-100
101-150
151-200
201-250
>250
C
Treated WGV (egg)
grapes (C-1)
wood
meadow
meadow
alfalfa
hazelnut trees
grapes (C-2)
D
Treated WGV (egg)
grapes (C-1)
wood
meadow
meadow
alfalfa
hazelnut trees
grapes (C-2)
0 30 60 120 180 240
metres

Figure 6


Treated WGV (milk)
A
orchard
grapes
hazelnut trees
Treated WGV (egg)
Treated WGV (milk)
B
orchard
grapes
hazelnut trees
Treated WGV (egg)
Treated WGV (milk)
C
orchard
grapes
hazelnut trees
Treated WGV (egg)
Classes
0
1-2
3-5
6-10
11-20
21-50
51-100
101-150
151-200
201-250
>250
Treated WGV (milk)
D
orchard
grapes
hazelnut trees
Treated WGV (egg)
Treated WGV (milk)
E
orchard
grapes
hazelnut trees
Treated WGV (egg)
Treated WGV (milk)
F
orchard
grapes
hazelnut trees
Treated WGV (egg)
0 25 50 100 150 200
metres